Interferon-α Production by Plasmacytoid Dendritic Cells Is Dispensable for an Effective Anti-Cytomegalovirus Response in Adaptor Protein-3-Deficient Mice. [Del Prete A.*, Luganini A.* co-first author]

IFN-α production by plasmacytoid dendritic cells is dispensable for an effective anti-cytomegalovirus response in AP-3-deficient mice

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ABSTRACT

Adaptor protein-3 (AP-3) is a hetero-tetrameric complex, which regulates vesicular trafficking. Mutations of the β3A subunit cause the Hermansky-Pudlak syndrome type 2 (HPS-2), a rare genetic disease characterized by albinism, platelet defects and recurrent infections. Likewise, *pearl* mice, which lack functional AP-3, show several HPS-2 defects. AP-3 absence results in defective TLR trafficking and signalling in dendritic cells (DC), but its effect on the efficiency of the *in vivo* antiviral response is unclear. We evaluated the impact of AP-3 deficiency on the distribution of DC subsets, IFN production, and the susceptibility to murine cytomegalovirus (MCMV) infection. *Pearl* mice showed a distribution and frequency of conventional (cDC) and plasmacytoid DC (pDC) similar to that of wt mice both before and after MCMV infection. Moreover, *pearl* mice controlled MCMV infection even at high virus doses and showed a normal production of IFN-α. Since pDC, but not cDC, from *pearl* mice showed an impaired IFN-α and TNF-α production in response to prototypic DNA (MCMV and HSV) or RNA (VSV) viruses *in vitro*, it is likely that MCMV infection can be controlled *in vivo* independently of an efficient production of IFN-α by pDC, and that AP-3 complex has a minimal impact on protective antiviral responses.

Adaptor complexes (AP-1 through 5) are hetero-tetrameric proteins involved in distinct intracellular vesicular transport pathways. AP-3 escorts proteins from the early trans-Golgi network to lysosome-related organelles (Dell'Angelica et al 2009; Feng et al 1999). A mutation in the gene *ADTB3A*, which encodes for the β3A subunit, causes the Hermansky-Pudlak syndrome type 2 (HPS-2), an autosomal recessive inherited disease characterized by partial albinism, prolonged bleeding and immunodeficiency (Badolato et al 2007).

Interestingly, human HPS-2 has its murine counterpart in the *pearl* strain of mice, which bear a mutation in the *AP3B1* gene, the mouse orthologous of *ADTB3A*, and share many aspects of the human disease (Feng et al 1999).

Being responsible for protein trafficking to lysosomes, AP-3 affects important immune processes. In fact, both AP-3-deficient mice and HPS-2 patients show abnormalities involving the polarization of lytic granules inside cytotoxic T cells and the intracellular localization of CD1b and CD1d, which are required for the presentation of lipid antigens and NKT cell development (Elewaut et al 2003). AP-3 has been linked to TLR signalling in dendritic cells (DC) and is required for TLR4 recruitment to phagosome and MHCII presentation of antigens internalized by phagocytosis (Mantegazza et al 2012). Furthermore, AP-3, as well as the other HPS proteins, is required for plasmacytoid DC (pDC) signalling through TLR7 and TLR9 (Blasius et al 2010; Sasai et al 2010). These facts point out the involvement of AP-3 in the development of efficient innate and adaptive immune responses. DC play a pivotal role in the response to a wide variety of viruses both in humans and mice, including cytomegalovirus (CMV) (Rahman et al 2011, Rölle and Olweus 2009). The experimental model of murine CMV (MCMV) infection greatly advanced our understanding of the DC role and, due to the many features shared with the species-specific human counterpart virus (HCMV), it is widely used to investigate the innate and adaptive immune responses to CMV *in vivo* (Krmpotic et al 2003; Riera et al 2000). In this regard, it has been established that conventional DC

(cDC) support productive MCMV infection both *in vitro* and *in vivo* (Andrews et al 2001), but they also have an important role in the control and clearance of viral infection. After infection, CD11b DC secrete innate immunity cytokines, including IFN-α, and are involved in NK cell activation. pDC are considered the main producers of IFN-α and IFN-β in response to MCMV infection in a TLR9- and MyD88-dependent manner (Krug et al 2004). However, several studies showed that, in addition to pDC, other cell types, such as cDC, macrophages and lymphoid-tissue stromal cells, can produce IFN-α in response to *Herpes Simplex* virus (HSV) or CMV infections. Moreover, IFN-α production by non-pDC is largely TLR9-independent and responsible for most of IFN-α produced (Hochrein et al 2004; Sozzani et al 2010). Although AP-3 protein is required for TLR9 and TLR7 activation in pDC, it is unclear whether the *pearl* mice defect can influence the *in vivo* immune response to viral infections. The purpose of this study was to determine the degree of susceptibility of *pearl* mice to MCMV infection and to assess whether MCMV infection modulates DC subsets and IFN production in these mice.

In order to characterize DC, CD34+ bone marrow (BM) positive cells from *pearl* mice (kindly provided by Prof. Dr. Stephan Ehl) and C57BL/6J wt mice (Charles River Breeding Laboratories) were purified by positive immunoselection and cultured with mGM-CSF (40 ng/ml) and Flt-3L (100 ng/ml) to generate cDC, or with Flt3L (200ng/ml) only to generate pDC (Del Prete et al 2007). As previously shown (Sasai et al 2010), *pearl* mice DC were comparable to wt DC in terms of surface expression of CD11c, CD11b, MHC I and II and CD80 (data not shown). Then, cDC and pDC from *pearl* mice were functionally characterized for cytokine production in response to different virus infections. Since TLR7 and TLR9 are required for pDC to sense viral nucleic acids, we investigated the response of AP-3-deficient pDC to prototypic DNA viruses, such as MCMV (Smith strain, ATCC VR.194) and HSV-2 (a clinical isolate), which stimulate pDC mainly through TLR9, and to a RNA virus, such as the *Vesicular Stomatitis* virus (VSV, serotype Indiana), which

triggers IFN-α production by pDC mainly through TLR7 (Ahmed et al 2009). To this end, pDC were infected with the different viruses at a MOI of 1, or treated as a positive control for TLR-9 stimulation, with CpG 2216 type-A (20 μg/ml) for 24 hours.

As shown in Fig. 1A, regardless of the virus, a severe defect in IFN-α production, both at the mRNA (left panel) and protein levels (middle panel), was observed in *pearl* pDC compared to wt pDC. A similar defect was observed in TNF-α secretion as well (Fig. 1A, right panel). In contrast, no significant differences in IFN-α levels were observed after MCMV stimulation of *pearl* cDC compared to wt cDC (Fig. 1B, left panel), and cDC from *pearl* mice were able to produce normal amounts of TNF-α after either MCMV or LPS stimulation (1 μg/ml) (Fig. 1B, right panel). Results reported in Fig.1 indicate that the AP-3 deficiency in *pearl* mice selectively impairs the ability of pDC, but not of cDC, to respond to different viruses that signal through TLR7 or TLR9.

Moreover, these observations suggest that AP-3 deficiency abolishes the TLR9-mediated IFN-α response of pDC during MCMV infection. This, in turn, may lead to an increased susceptibility to MCMV *in vivo*. In this regard, MCMV infection is considered a suitable experimental model to study systemic *Herpesvirus* infection, as well as the TLR-dependent and -independent mechanisms of type I IFN induction. In fact, in the host MCMV spreads through the blood reaching liver and spleen, which are the principal sites of viral replication, and induces a type I IFN response mediated by different cell-type-specific mechanisms of viral recognition and IFN expression (Steinberg et al 2009).

Mouse splenic DC include three main populations. The cDC splenic subsets can be identified on the basis of the presence or absence of a CD8α homodimer, while pDC are characterized by high B220 expression (Del Prete et al 2004). Both pDC and cDC play an important role in the antiviral response to MCMV: in addition, there is evidence that changes in their proportion can account for different susceptibility to viral infections (Andrews et al 2003;Traub et al 2012). In order to further

investigate whether the AP-3 deficiency affects the normal distribution of different splenic DC populations, we characterized the DC subsets for specific cell surface markers (Fig. 2A). DC were derived from wt and *pearl* mice spleens under steady-state conditions or after infection with MCMV (36 hours following challenge with $3x10^5$ PFU i.p.). As shown in Fig. 2B, the phenotypic characterization of spleen-derived DC from *pearl* and wt mice revealed similar proportions of CD11c+ DC (upper left panel), and comparable frequencies of cDC, both CD11b+CD8α- (upper right panel) and CD11b-CD8α+ (lower left panel), and of B220+ Siglec-H+ pDC (lower right panel). In addition, *pearl* mice maintained a stable population of CD11c+ cells following MCMV infection and no significant differences were found in the frequency of CD11b+CD8α-, CD11b-CD8α+, and B220+Siglec-H+ (Fig. 2B).

The AP-3 complex is also required for normal trafficking of CD1d, a molecule involved in the presentation of lipid antigens to NKT cells and in their selection. CD1d is constitutively expressed on CD11c+ cells and is reduced during virus infection (Lin et al 2005). In *pearl* mice, the abnormal trafficking of CD1d leads to increased expression of CD1d on the surface of different cell types and to a decreased expression in late endosomes coupled with a decreased NKT cell number (Elewaut et al 2003). In agreement with these data, FACS analysis showed a more than two-fold increase in CD1d expression levels on the surface of total splenic *pearl* mice-derived DC compared to wt cells (Fig. 2C upper and lower left panels). However, MCMV infection strongly reduced CD1d expression in *pearl* mice to levels comparable to those observed in wt mice (Fig. 2C upper and lower right panels).

To assess the effects of AP-3 deficiency in the cytokine response to *in vivo* MCMV infection, levels of IFN-α, TNF-α and IFN-γ were evaluated by ELISA in the sera of wt and *pearl* mice 36 hours after MCMV challenge ($3x10^5$ PFU, i.p.), when cytokine production exhibits a sharp peak (Orange et al 1996). According to several reports (Dalod et al 2003; Delale et al 2005; Swiecki et al 2010,

Zucchini et al 2008a), pDC are essential for the *in vivo* production of IFN-α during the initial stages of MCMV infection. However, we did not find any difference in the circulating levels of IFN-α in *pearl* compared to wt mice at 36 hours (Fig. 3A upper left panel), despite the defective IFN-α production observed in vitro in *pearl* mice-derived pDC stimulated with MCMV (Fig. 1A). As shown in Fig. 3A (upper right panel), similar levels of IFN-α were also detected at a later time point (48 hours), a time in which IFN-α production is considered non pDC-dependent (Delale et al 2005; Swiecki et al 2010). A mild non-significant decrease in TNF-α production was observed in *pearl* mice compared to wt (Fig. 3A lower left panel). IFN-γ levels were evaluated in the sera of MCMV-infected mice as well as in mice injected with the glycolipid antigen α-galactosylceramide (α-GalCer) (100 ng/ml), which induces NKT cell activation in a CD1d-dependent manner (Kawano et al 1997). Similarly to IFN-α, *pearl* mice were as efficient as wt animals in IFN-γ production following MCMV infection. In contrast, the IFN-γ levels were strongly reduced in α-GalCer-treated *pearl* mice compared to wt (Fig. 3A lower right panel), in agreement with the occurrence of a reduced number of Vα14 invariant NK T (Vα14i NK T) cells, which has previously been shown in *pearl* mice (Elewaut et al 2003).

To determine the overall susceptibility to MCMV infection, AP-3-deficient mice were challenged with different viral doses. The mortality and viral load in different organs were assessed in comparison with those of wt mice. Upon infection with $1x10^5$ or $3x10^5$ PFU, all wt and AP3-deficient mice survived (Fig. 3B). At the higher dose, $5x10^5$ PFU, survival of both wt and AP-3-deficient mice was approximately 80% (Fig. 3B). The viral titres in multiple tissues of *pearl* and wt mice infected with different doses of MCMV (1, 3 or 5 x 10^5 PFU) were assessed at different post-infection (p.i.) time points. As shown in Fig. 3C, in the liver of *pearl* and wt mice similar levels of MCMV were titrated at 2 days p.i. Since MCMV replicates in salivary glands during the late stage of infection, the viral titres were measured in salivary glands on day 15 p.i., and no differences were

found. Determination of viral titres in liver, lung and spleen on day 15 p.i., revealed that *pearl* mice, as wt animals, were able to reduce MCMV loads, regardless of the dose, below detection limit.

Taken together, these observations suggest that *pearl* mice, despite a major defect affecting the *in vitro* pDC response to TLR7 and TLR9 stimuli, produce normal levels of IFN-α following MCMV infection and control the infection with the same efficiency as wt animals.

The role of pDC in the early innate control varies according to the virus and the infection model (Cervantes-Barragan et al 2012). Several reports explored the role of pDC and IFN-α production in response to MCMV infection, by using different models of pDC depletion or functional impairment in the absence of the TLR9/MyD88 pathway. For example, pDC depletion in Ikaros$^{L/L}$ mice (Allmann et al 2006) or BDCA2-DTR transgenic mice (Swiecki et al 2010) resulted in deficient production of IFN-α at early time points after MCMV infection. However, IFN-α production was restored at later time points (Allmann et al 2006; Swiecki et al 2010) by cells other than pDC. The effects of pDC depletion on the overall anti-MCMV response seem to be dependent on viral doses. In fact, at high viral loads, BDCA2-DTR mice, similarly to what we observed in *pearl* mice, were efficient in containing MCMV replication (Swiecki et al 2010). A less efficient control of viral replication in the Ikaros$^{L/L}$ pDC depletion model may be due to the potential impairment of other hematopoietic lineages (Allmann et al 2006).

The role of TLR9 in MCMV infection, in the presence or in the absence of pDC, has been carefully characterized by Delale et al (2005). Interestingly, these authors observed that TLR9-/- mice maintained a pDC-dependent IFN-α production, in response to MCMV infection, notwithstanding the *in vitro* defective IFN-α secretion. These findings, in line with our data, suggest that some compensatory mechanisms may occur during the *in vivo* response to MCMV infection. It is therefore conceivable that in *pearl* mice other haematopoietic and non haematopoietic cells, (i.e., lymphoid-tissue stromal cells) could contribute to the overall *in vivo* production of IFN-

α (Andoniou et al 2005; Delale et al 2005). In this regard, it has been shown that cDC are able to produce type I IFN in a TLR9-independent manner, and that TLR2 and TLR3 may play a role in the defence against MCMV infection (Tabeta et al 2004; Barbalat et al 2009). Moreover, a TLR-independent production of IFN-α by cDC in response to MCMV has been reported to occur through the retinoic acid-inducible gene 1 (RIG-1) (Yoneyama et al 2004). Interestingly, although TLR7 mediates recognition of ssRNA virus and TLR9 sense MCMV, Zucchini et al reported that TLR7 and TLR9 redundantly affect IFN-α production by pDC during MCMV infection *in vivo*. These authors observed a decrease in IFN-α production in TLR7/9 double deficient mice that however was not detected in TLR9-/- mice (Zucchini et al 2008b). This is in agreement with our and Delale's data, and further underlines the occurrence of TLR9-independent mechanisms. However, unlike TLR9-/- mice and TLR7/9 double-deficient mice, which were more susceptible to MCMV infection especially at high challenging doses (Delale et al 2005; Zucchini et al 2008b), we observed that *pearl* mice did not show an increased susceptibility to MCMV challenge when compared to wt controls. This difference may stem from the different players that had been knocked out in the various experimental mouse models (i.e., AP-3 versus TLR9). The AP-3 complex interacts with cleaved TLR9 and facilitates its trafficking to LAMP2 lysosome-related organelles (LRO), whereby TLR9 can engage TRAF3 and IRF7 and induce transcription of IFN genes. However, in AP-3 deficient cells, TLR9 is unable to traffic to a specialized $LAMP2^{+}$ LRO and to engage IRF7, but enters NF-kB endosome becoming competent to activate certain pro-inflammatory cytokine genes (Sasai et al 2010). Thus, it is conceivable that the ability of TLR9 to sense MCMV DNA and to stimulate the expression of inflammatory cytokines, even in AP-3-deficient mice, can account for the initiation of an antiviral response that leads to the control of MCMV infection. Furthermore, the observation of an efficient anti-MCMV response in AP-3-deficient mice is in agreement with data

from Jessen et al, who reported that *pearl* mice infected with lymphocytic choriomeningitis virus (LCMV) control infection and clear the virus from all organs (Jessen et al 2013).

In conclusion, our results with the AP-3-deficient mouse model, while confirming previous results obtained with different experimental models on the limited role of pDC in contributing to the overall response to MCMV infection, support the view that the impaired immune functions caused by mutation in the *ADTB3A* gene have a minimal impact on the generation of protective antiviral responses.

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FIGURE LEGENDS

Figure 1. BM-derived pDC from *pearl* mice are defective for IFN-α production in response to virus infections. CD34+ BM positive cells from *pearl* and C57B6/L wt mice were purified by positive immunoselection and treated to generate cDC or pDC as previously described (Del Prete and others 2004). pDC were infected at MOI of 1 with MCMV, HSV2, VSV or treated with CpG ODN 2216 (CpG-A) (20μg/ml). **(A, left panel)** After 24 hours, total RNA was extracted, retrotranscribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc.) and Real-time PCR performed with iQ^{TM} SYBR Green Supermix (Bio-Rad Laboratories Inc.) was carried out using primers specific for IFN-α (sense, 5'-CCACAGGATCACTGTGTACCTGAGA -3'; antisense, 5'-CTGATCACCTCCCAGGCACAG -3'). The cDNA were amplified for 45 cycles (95°C for 10 s and 60°C for 30 s). Results were normalized to the 18S signals and are presented as fold increase relative to the mRNA levels in unstimulated cells (assumed as the 1.0 value) (one representative experiment out of three). **(A, center and right panels)** IFN-α and TNF-α concentrations were measured by VeriKine Mouse IFN-α ELISA (PBL Interferon Source), and Duoset ELISA (R&D System) in the supernatants from BM-derived pDC stimulated as described above. **(B)** IFN-α and TNF-α concentrations were measured in the supernatants from BM-derived cDC infected with MCMV (MOI 1) or treated with LPS (1μg/ml) for 24 hours. Data are expressed as means ± SEM (n=3). Results were analyzed by two-tailed Student t-test. $^{*}p < 0.05$.

Figure 2. Phenotypic characterization of DC subsets in *pearl* mice under steady-state conditions or following MCMV infection.

Wt and *pearl* mice were left untreated or infected i.p. with $3x10^5$ PFU of MCMV, and spleens were harvested 36 hours later. **(A)** Splenocytes were analyzed by FACS CANTO II System (BD Biosciences) for the expression of CD11c, NK 1.1, CD11b, CD8α, B220 (all from BD Biosciences), Siglec-H (eBioscience). The FACS gating strategy used is shown. Among the total DC (CD11c+), cDC were identified by the phenotypes CD11b+CD8α- and CD11b-CD8α+, and pDC by the phenotype B220+ Siglec-H+.

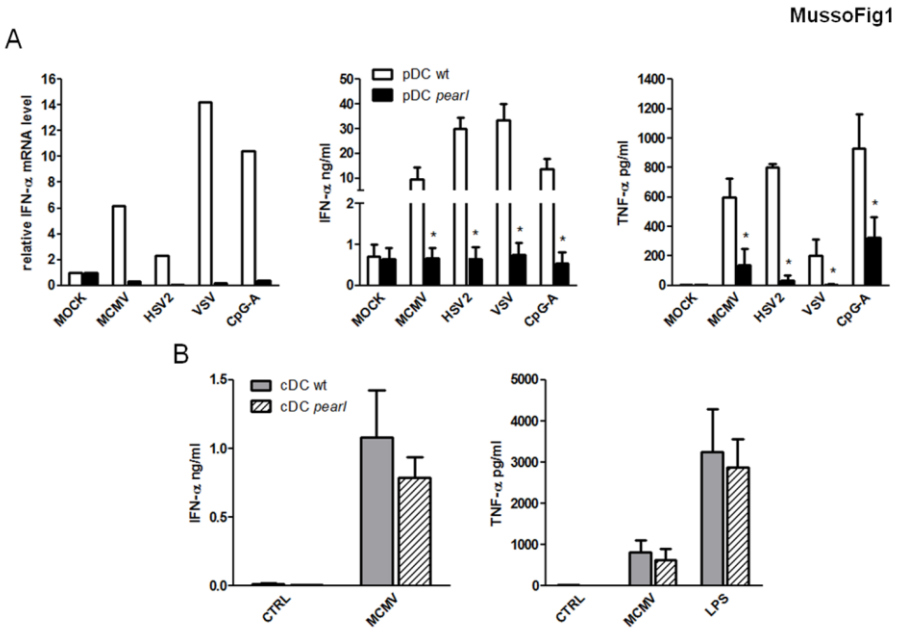
(B) Frequencies for each population in the spleens of infected and uninfected mice are shown. Data are presented as means ± SEM (bars show averages of four mice per group and one of 2 independent experiments is shown.)

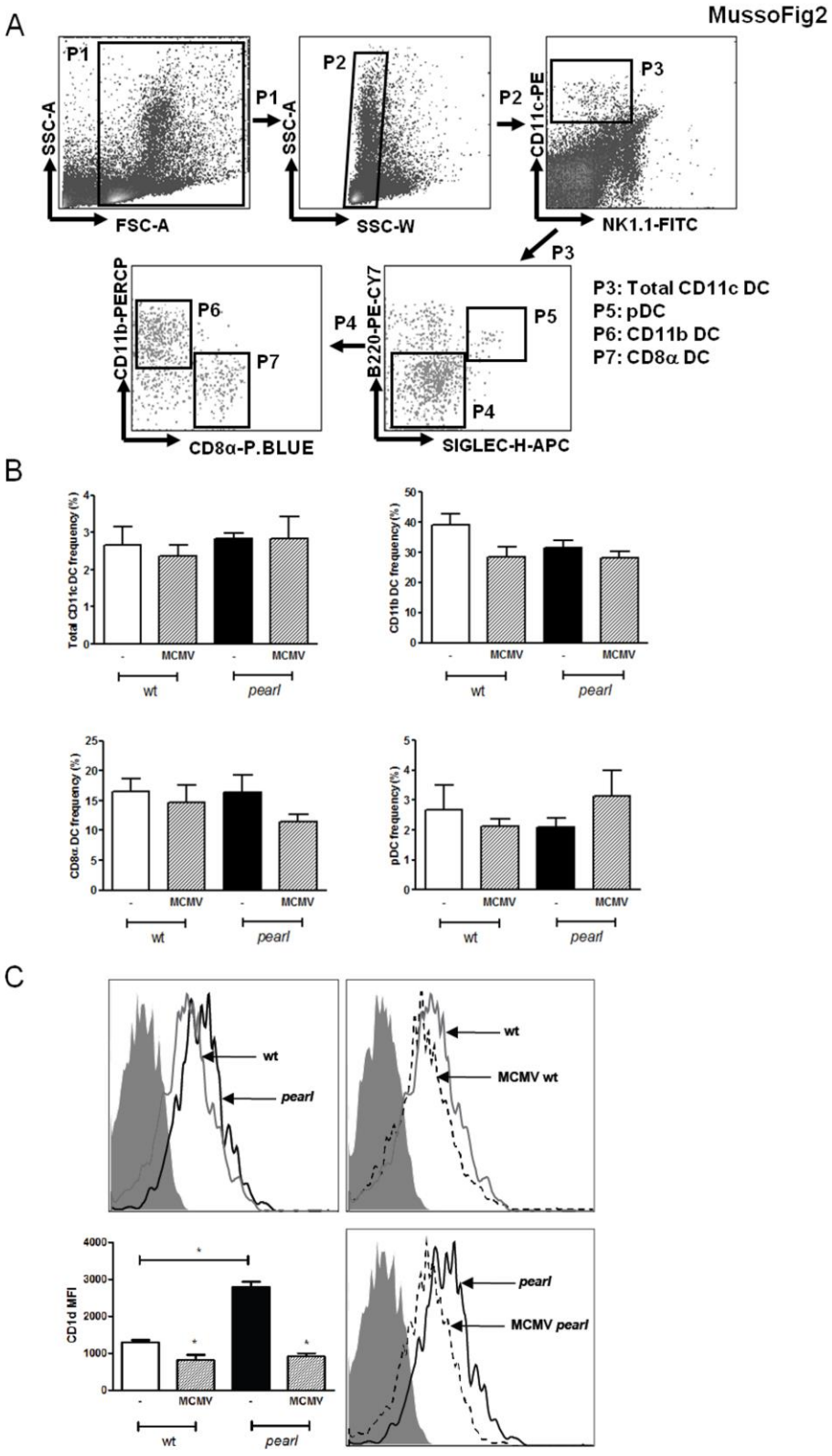
(C) Expression of cell-surface CD1d by CD11c+ cells from spleens of uninfected and MCMV-infected wt and *pearl* mice. Histograms are representative plots of two independent experiments of four mice per group. Isotype control IgG is shown as grey filled histograms, untreated wt mice as black line and untreated *pearl* mice as red line, MCMV-infected wt and MCMV-infected *pearl* mice as blue line. Mean fluorescence intensity (MFI) of CD1d on CD11c+ cells from the spleen of uninfected or MCMV-infected wt and *pearl* mice is shown as means ± SEM (n=4) **(C low left panel)**. Results were analyzed by two-tailed Student t-test. $^{*}p < 0.05$.

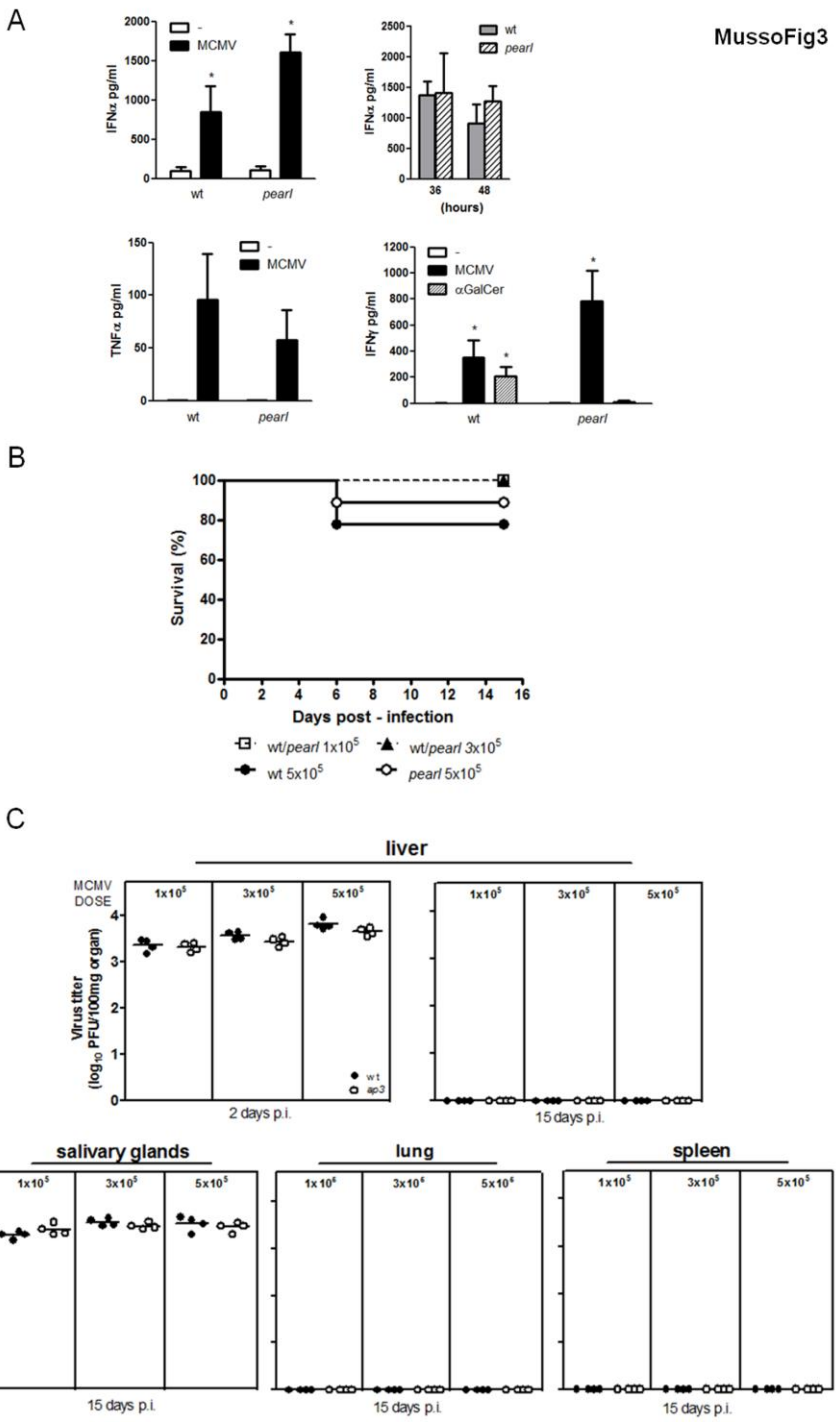
Figure 3. Cytokine production, survival and viral load in wt and *pearl* mice during MCMV infection.

(A) Serum cytokines in wt and *pearl* mice during MCMV infection with $3x10^5$ PFU of a salivary gland stock virus i.p. injected. IFN-α (left upper panel) and TNF-α (lower left panel) serum levels

were measured by ELISA at 36 hours. Values are expressed as means ± SEM of two independent experiments (four mice per group). Circulating IFN-α levels of infected mice were also determined at later time point (48 hours) (upper right panel) in two independent experiments (three mice per group). Wt and *pearl* mice were infected with MCMV or injected with α-GalCer (100 ng/ml) and analyzed for IFN-γ serum levels at 36 hours by ELISA (lower right panel). Data are combined from two independent experiments (four mice per group). **(B)** Survival curves of wt and *pearl* mice infected with MCMV. Nine wt or *pearl* mice were infected i.p. with $1x10^5$, $3x10^5$ or $5x10^5$ PFU, and monitored daily for a period of 15 days. The Kaplan-Meier survival analysis model was used to assess the survival of mice in each group. As indicated, the mortality was presented as the percentage of survival mice. **(C)** Virus production in wt and *pearl* mice infected with MCMV. The graph represents the virus titres in four mice for group infected i.p. with 1×10^5, 3×10^5 or 5×10^5 PFU of MCMV. The viral loads were evaluated in the liver at 2 and 15 days p.i, and in salivary glands, lungs, and spleens at 15 days p.i. All organs were individually weighed, homogenized in DMEM-10% FBS as a 10% (w/v) suspension and centrifuged at 12000 g x 15' at 4 °C. Supernatants were titrated by plaque assay on NIH3T3 cells and given values were calculated for 100 mg of each organ. Results were analyzed by two-tailed Student t-test. *$p < 0.05$.


MussoFig1
A
relative IFN-α mRNA level
MOCK
MCMV
HSV2
VSV
CpG-A
pDC wt
pDC pearl
IFN-α ng/ml
TNF-α pg/ml
B
cDC wt
cDC pearl
CTRL
MCMV
LPS


MussoFig2
A
P1
SSC-A
FSC-A
P2
SSC-W
P3
CD11c-PE
NK1.1-FITC
P4
B220-PE-CY7
SIGLEC-H-APC
P5
P6
CD11b-PERCP
P7
CD8α-P.BLUE
P3: Total CD11c DC
P5: pDC
P6: CD11b DC
P7: CD8α DC
B
Total CD11c DC frequency (%)
CD11b DC frequency(%)
CD8α DC frequency (%)
pDC frequency(%)
MCMV
wt
pearl
C
wt
pearl
MCMV wt
MCMV pearl
CD1d MFI


MussoFig3
A
IFNα pg/ml
MCMV
wt
pearl
(hours)
TNFα pg/ml
IFNγ pg/ml
αGalCer
B
Survival (%)
Days post - infection
wt/pearl 1x10^5
wt/pearl 3x10^5
wt 5x10^5
pearl 5x10^5
C
liver
MCMV DOSE
Virus titer (log10 PFU/100mg organ)
2 days p.i.
15 days p.i.
salivary glands
lung
spleen